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REVIEW

Emergency Services of Viral RNAs: Repair and Remodeling

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SUMMARY Reproduction of RNA viruses is typically error-prone due to the infidelity of their replicative machinery and the usual lack of proofreading mechanisms. The error rates may be close to those that kill the virus. Consequently, populations of RNA viruses are represented by heterogeneous sets of genomes with various levels of fitness. This is especially consequential when viruses encounter various bottlenecks and new infections are initiated by a single or few deviating genomes. Nevertheless, RNA viruses are able to maintain their identity by conservation of major functional elements. This conservatism stems from genetic robustness or mutational tolerance, which is largely due to the functional degeneracy of many protein and RNA elements as well as to negative selection. Another relevant mechanism is the capacity to restore fitness after genetic damages, also based on replicative infidelity.

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Conversely, error-prone replication is a major tool that ensures viral evolvability. The potential for changes in debilitated genomes is much higher in small populations, because in the absence of stronger competitors low-fit genomes have a choice of various trajectories to wander along fitness landscapes. Thus, low-fit populations are inherently unstable, and it may be said that to run ahead it is useful to stumble. In this report, focusing on picornaviruses and also considering data from other RNA viruses, we review the biological relevance and mechanisms of various alterations of viral RNA genomes as well as pathways and mechanisms of rehabilitation after loss of fitness. The relationships among mutational robustness, resilience, and evolvability of viral RNA genomes are discussed.

KEYWORDS RNA viruses, evolvability, fitness, mutational tolerance, repair, replication fidelity, robustness

INTRODUCTION

Replicative systems of RNA viruses are typical error-prone, primarily due to the low fidelity of their RNA-dependent RNA polymerases (RdRP) and the lack (in the overwhelming majority of cases) of proofreading mechanisms. Although the extents of replicative infidelity vary among different RNA viruses, the purified picornaviral RdRPs generally demonstrate high levels of nucleotide misincorporation, especially in the case of transitions, which sometimes have a rate as high as $\sim 10^{-4}$ per site per template copy (1–6). These values obviously depend on the experimental conditions and do not necessarily accurately correspond to the real situations in infected cells (which also vary). Nevertheless, taking into account that, for example, picornaviral genomes consist of $\sim 10^4$ nucleotides (nt), such a level of enzyme infidelity is consistent with the acquisition, on average, of one mutation by each newly synthesized molecule of RNA of these viruses. Indeed, a recent study directly demonstrated that genomes of the progeny of a clone of poliovirus had, on average, more than two mutations each after merely two passages in tissue culture (7).

Interestingly, such replicative negligence is not an obligatory feature of RdRPs. Indeed, even a point mutation may increase the accuracy of these enzymes severalfold (8–12; also see the other references in this paragraph). However, a more faithful replication may result in a fitness loss by preventing accumulation of adaptive mutations (13–22), although the relationships between fidelity and fitness may differ between virus-host systems, being influenced in part by the conditions under which the fitness assay is performed (23–29). Moreover, a decrease in replicative fidelity may also lead to fitness loss, suggesting that the accuracy of replication of RNA viruses is naturally fine-tuned (29).

Due to replicative infidelity, populations of these viruses are highly heterogeneous, i.e., they exist as quasispecies (30–34). Importantly, the level of heterogeneity depends not only on the intrinsic fidelity of the viral RdRPs but also on the mode of genome replication (35). Two distinct mechanisms, the "stamping machine" and "geometric replication" mechanisms, may be operating. The former implies that all the progeny genomes within a given cell are templated just by the infecting genome(s), whereas the latter posits that the newly synthesized genomes serve as templates as well, forming their own distinct "lineages." Obviously, the heterogeneity of the final harvest of the infected cell should be much larger in the case of geometric replication and should depend on the number of genome "generations" in this cell. Specifically, it was estimated that, on average, 5 generations of poliovirus RNA were produced in a single HeLa cell under the experimental conditions used (35). The mode of replication and number of genome generations vary in different virus-host systems, thereby affecting the size of the space of genome diversity.

In any case, heterogeneous populations are expected to include numerous low-fit or even dead genomes. Indeed, a severalfold increase in the error rate (leading to the appearance of a few additional mutations in newly synthesized viral genomes) may result in a total extinction—"error catastrophe"—of the relevant population (36–41).

Replicative infidelity may be especially relevant to fitness if it concerns small viral populations and various kinds of bottlenecks which viruses may encounter. In particular, such bottlenecks occur when the virus has to overcome various barriers, whether they are intrahost (e.g., blood-brain barrier and others) or interhost (42–45) barriers. On the one hand, overcoming various barriers may require a significant level of infidelity, leading in particular to the generation of adaptive mutations. On the other hand, if after overcoming such barriers infection is initiated by only a few or even a single low-fit infectious genome, the newly generated viral population may be more or less seriously invalidated or deadlocked altogether, a phenomenon known as the Muller ratchet (46–49).

Notably, bottlenecking effects have an important but very poorly understood aspect. It is known that a single infectious dose in tissue cultures, e.g., a PFU of poliovirus, may contain as many as hundreds of virions. Although some of these virions may carry dead genomes, there is no reason to believe that such genomes constitute a majority, let alone the overwhelming one. Hence, only a minute minority of the potentially infectious genomes invading target cells appears to have the chance to initiate viral reproduction. Very little is known about the nature and factors affecting this phenomenon, except that the specific infectivity of viruses or viral RNA (number of virions or RNA molecules able to start productive infection) may vary by several orders of magnitude, depending on the primary structure of the genome (50–53), and also varies for different host cells. The number of physical particles needed to avoid a fitness decrease due to a natural bottleneck is generally unknown, although these values may be quite relevant.

Notwithstanding these circumstances, RNA viruses demonstrate a remarkable potential for genetic and structural conservation under constant conditions. For example, sequences of portions of the genomes of several poliovirus strains studied in our lab (54, 55) turned out to be nearly identical to the sequences of these strains grown independently for nearly 3 decades in other countries (56–60). Of course, such identity concerned the consensus (i.e., averaged) sequences. Under natural conditions, the primary structure of the genomes of RNA viruses undergoes more or less marked alterations, both neutral and adaptive, but the identity of the virus as belonging to a distinct type or species is usually retained. Much more rarely, sharp qualitative changes and generation of new viral taxa may also occur.

Somewhat paradoxically, both genetic conservatism and evolvability are based, to a significant extent, on replicative infidelity. When, for any reason, new infection is initiated by a debilitated viral variant, there is often the possibility of regaining the original fitness through acquisition of reversions or compensatory mutations. On the other hand, population heterogeneity includes viral variants that, regardless of their fitness in a constant environment, exhibit enhanced fitness if the population meets unfavorable conditions, e.g., innate or adaptive immunity, antiviral drugs, unfamiliar hosts, etc. For example, polioviruses are highly sensitive to the inhibitory effect of millimolar concentrations of guanidine hydrochloride. However, populations of this very sensitive virus always contain tiny proportions (depending on the drug concentration tested) of guanidine-resistant (g^r) mutants (61), or even mutants whose growth requires the presence of this drug (62; also see below). The presence of drug-resistant and drug-dependent variants in largely sensitive populations has also been reported for other picornaviruses and other inhibitors (63–71). Different variants within heterogeneous viral populations may complement each other in performing some functions, as appears to be the case with poliovirus (10, 14, 22, 32), although the converse situation, negative *trans*-dominance of debilitating mutations, has also been documented (72, 73).

Here we consider numerous ways in which RNA viruses overcome or mitigate negative effects of their replicative infidelity (and of genome-damaging environmental factors) and discuss why RNA viruses are remarkably robust despite their replicative infidelity and why they exhibit a remarkable evolvability despite their robustness. We focus predominantly on picornaviruses, one of the most extensively characterized viral

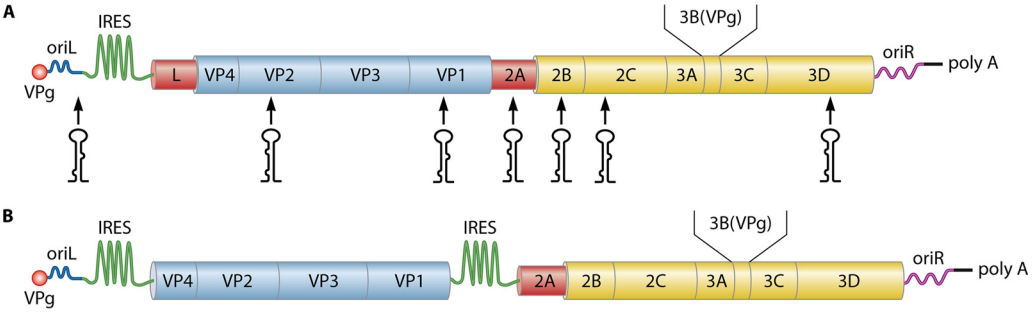

FIG 1 Schematic representation of the genomes of picornaviruses. (A) Genome of monocistronic (most prevalent) picornaviruses. The leader L and 2A proteins (shown in red and not to scale) are highly variable in structure and function and may be dispensable for viral viability. They may be represented by several distinct copies or be absent in a given genome. The various positions of the replicative *cre* (*ori*l) *cis*-element (hairpins) are indicated. The VPg protein covalently linked to the 5′ end of the viral RNA is shown as a red circle. (B) Genome of dicistronic picornaviruses. The position of *cre* is unknown.

families, but some additional lessons derived from the behavior of certain other RNA viruses are also considered. The regularities emerging from this analysis seem to be applicable to RNA viruses in general.

PICORNAVIRUSES AND THEIR GENOMES

Picornaviruses (representatives of the *Picornaviridae* family) are small (roughly 30 nm in diameter), nonenveloped, icosahedral animal viruses which are classified into >35 genera, ~80 species, and hundreds of types and include pathogens causing important human and animal diseases, such as poliomyelitis, hepatitis A, common cold, myocarditis, encephalitis, foot-and-mouth disease, and many others (www.ictv.global/report/picornaviridae). They have a single-stranded RNA genome of positive polarity (i.e., translatable) containing ~6,700 to 10,100 nt. This genome (Fig. 1A) carries a single open reading frame (ORF) that encodes a polyprotein, which is eventually processed into about a dozen "mature" functional proteins. The polyprotein is usually considered a modular structure composed of the following three parts: P1, containing capsid proteins VP1 to -4 and, in some viruses, also the leader protein L; P2, containing nonstructural proteins 2A, 2B, and 2C; and P3, containing nonstructural proteins 3A, 3B (or VPg, the RNA-priming protein), $3C^{pro}$ (protease), and $3D^{pol}$ (RdRP) (74, 75). As far as picornaviral proteomics is concerned, the most striking variability is exhibited by the nonstructural proteins L and 2A, which are involved mainly (though not exclusively) in the interaction with cellular innate immunity and generally are not essential for viral viability. They may be regarded as accessory or "security" proteins (76). Picornaviruses differ from one another not only with respect to the structure and function of these proteins but also by having various numbers of distinct molecular species: 0 to 2 for L (if L*, encoded by another ORF of the genomes of some cardioviruses, is also counted) and up to 5 for 2A (76, 77).

The genomes of some picornaviruses also have additional, relatively small overlapping ORFs (78–80). In certain cases, the relevant proteins were shown to perform "security" functions, and it may be supposed that still-uncharacterized such proteins are mainly dedicated to the same profession. It may also be noted that picornaviruses have been described in which the proteins characteristic of this viral group are encoded in two separate ORFs, corresponding to P1 and P2-P3, respectively (81, 82) (Fig. 1B). Furthermore, enteroviruses of the G species which contain a new gene at the 2C/3A junction, encoding a predicted papain-like protease similar to that of coronaviruses, were recently isolated (83, 84). Also, the existence of a potential second ORF downstream of the entire main polyprotein-encoding sequence was reported (85). Still another possible pathway for evolutionary diversification of picornaviruses, genome segmentation, was demonstrated experimentally (see below). These examples illustrate the evolutionary potential of picornaviruses.

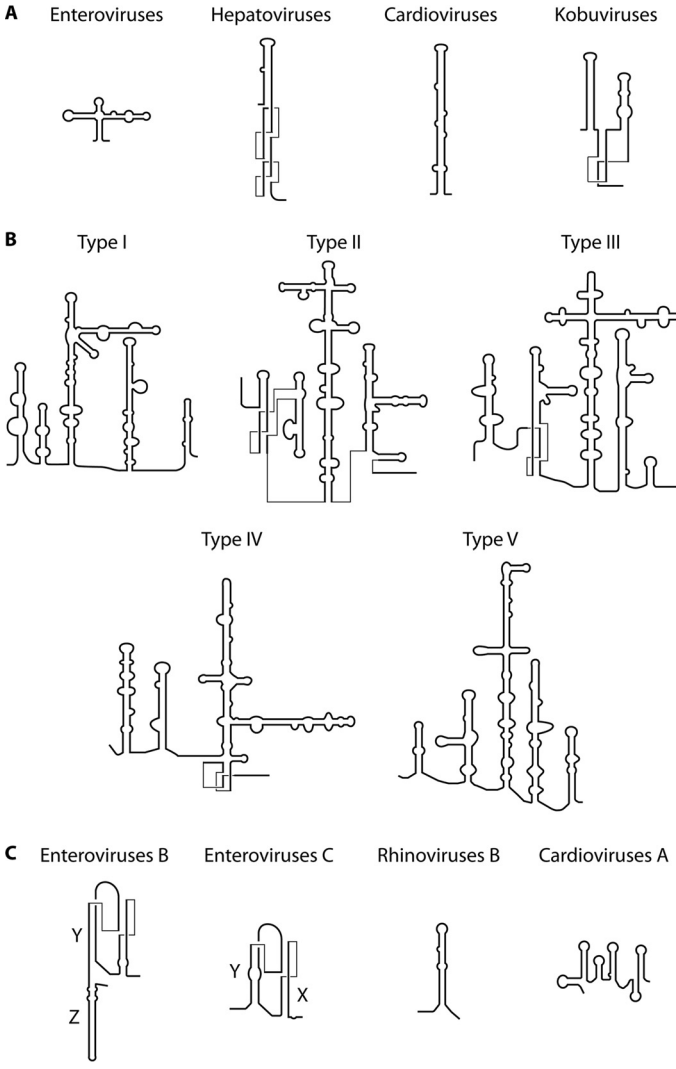

FIG 2 Examples of structural variability of the key *cis*-elements of picornavirus genomes. (A) *ori*Ls of enteroviruses (as exemplified by this element in poliovirus type I), hepatoviruses (hepatitis A virus), cardioviruses (mengovirus), and kobuviruses (aichivirus). (B) IRES elements of type I (poliovirus type 1), type II (Saffold virus), type III (hepatitis A virus), type IV (HCV-like; bat hepatovirus), and type V (aichivirus). (C) Examples of different *ori*Rs. Thin lines in all panels are used to link distinct structural elements and are not depicted to scale.

The polyprotein-encoding part of picornavirus genomes is flanked by 5′ and 3′ untranslated regions (5′ UTR and 3′ UTR) containing key *cis*-elements that vary markedly in structure in different picornaviral genera but perform similar functions, i.e., replicative (*ori*L and *ori*R; in the 5′ UTR and 3′ UTR, respectively) and translational (internal ribosome entry site [IRES]; predominantly in the 5′ UTR but also in the intercistronic region of bicistronic representatives) functions. The functional and structural features of these elements vary tremendously among different picornavirus genera (86, 87) (Fig. 2), and it is reasonable to briefly characterize them, especially those that are considered often.

The ~90-nt enterovirus *ori*L has a cloverleaf-like structure (88, 89) (Fig. 2A). This element plays multiple roles in viral reproduction, mainly through promoting formation of a complex ribonucleoprotein (RNP) structure involving several viral and host proteins (89–93). An essential component of this complex is the viral RdRP ($3D^{pol}$), which is recruited there in the form of its precursor, 3CD, i.e., covalently linked to the viral protease ($3C^{pro}$) (91). This *ori*L-3CD interaction involves the hairpin domain *d* of *ori*L and

the $3C^{pro}$ moiety of 3CD, as originally demonstrated by Andino et al. (90, 94). The interaction between these ligands is important for the initiation of the synthesis of both the viral (positive) and complementary (negative) RNA strands (89, 93, 95–98). It was recently shown that (at least in the case of coxsackievirus B3 [CVB3]) *ori*L, which is required for efficient genome replication, is not indispensable for viral viability: its removal does not kill the virus but rather decreases the efficiency of its RNA replication $\sim 10^5$-fold (99). It should be noticed, however, that the nonessentiality of *ori*L has so far been shown only for this particular virus (see also a reservation at the end of this section). *ori*Ls of picornaviruses other than enteroviruses exhibit great variability (some examples are presented in Fig. 2A), but their detailed and functional characterization has yet to be performed.

Initiation of translation of picornaviral RNA is accomplished via a cap-independent, IRES-dependent mechanism (100–103). Structures of IRES elements of different picornaviruses exhibit remarkable variations (86, 87, 104–110) and are represented by at least five structural types specific for this viral family or related to the IRES elements of certain flaviviruses (111, 112) (Fig. 2B). These translational *cis*-elements serve to bind ribosomes in order to create conditions for the initiation of translation (87, 107, 113). This process requires participation of both canonical initiation factors and IRES-specific (and cell-specific) *trans*-acting factors (ITAFs) (109, 114).

The structures of picornavirus *ori*Rs are rather variable (86) (Fig. 2C). Even within a single genus, *Enterovirus*, there are several distinct structural classes of this *cis*-element. In many enteroviruses, it is represented by a multidomain quasi-globular RNA structure maintained by a tertiary kissing interaction. Polioviruses and other representatives of enterovirus C species have two stem-loops, X and Y, while enteroviruses of species B have an additional stem-loop, Z, and human rhinoviruses have a single stem-loop (115–121). Picornavirus *ori*Rs are polyfunctional (122), being involved in negative RNA strand initiation (116, 119, 123), polyadenylation of the genomic RNA (124), and control of translation through (primarily but not only protein-mediated) interaction with the 5′ UTR, which results in noncovalent circularization of the genome (96, 125–130). The peculiarities of the *ori*R structures of various enteroviruses are in part responsible for their cell-specific fitness differences, which are especially prominent in neural cells, a fact which has direct relevance to viral pathogenicity (131–133).

It is noteworthy that enterovirus *ori*R, which is conserved and pivotal for efficient genome replication, is nevertheless not essential, as demonstrated by the viability (though with a markedly low fitness) of viruses lacking or having a severely damaged element (118, 123, 124, 134). On the other hand, deletion of a stem-loop of *ori*R of mengovirus (a cardiovirus) was reported to be lethal (135).

As mentioned above, in addition to their individual functions, the enteroviral 5′ UTR and 3′ UTR appear to interact with one another through the mutual affinity between proteins to which they bind, which results in noncovalent circularization of the picornavirus genome playing an important part in the control of viral translation.

The picornavirus genomes also contain an additional important replicative *cis*-element, *cre* (*cis*-acting replicative element) (136–138), also known as *ori*I. It folds into hairpin-containing structures (139, 140) that are located in different regions of the polyprotein ORF or in the 5′ UTR in different picornaviruses (86, 141) (Fig. 1A). The loop of its hairpin contains an oligoadenylate sequence which serves as the template for the uridylylation of the protein VPg (3B) by the viral RdRP (142, 143). The uridylylated form of VPg, $VPgpUpU_{OH}$, is the primer for initiation of the synthesis of viral RNA molecules (144–148). Again, mutational inactivation of *cre* may not kill the virus but rather decreases the efficiency of replication of its RNA $\sim 10^5$-fold, as reported for CVB3 (149). Note that *ori*L and *cre* elements of enterovirus genomes appear to work in cooperation, with both responsible for the correct VPg-pUpU-dependent initiation of the positive RNA strands. The loss of functional *cre* resulted in deletion of large 5′-terminal portions of CBV3 *ori*L and, reciprocally, deletion of *ori*L (which may occur upon chronic infection both in cultured cells and in diseased organisms [150–153]) was reported to make functional *cre* dispensable (149).

Picornavirus RNAs also have some additional conserved functional structures, such as the hairpin inhibiting the host antiviral enzyme RNase L (154, 155) as well as some other potentially biologically relevant elements with poorly characterized functions (156–158).

A separate question concerns the role and mechanism of generation of the 3′-terminal poly(A) tract. This tract is believed to play a role in the preservation of the genomic end, in particular by association with the poly(A)-binding protein (125, 159), which promotes the above-mentioned circularization of the viral RNA through protein-protein interaction. The poly(A) tract is synthesized by using the 5′-terminal poly(U) sequence in the negative viral RNA strands as the template, which in turn is templated by the poly(A) segment in the parental positive strand (160). It was noted, however, that the poly(A) stretches may be markedly longer than the poly(U) ones (161, 162).

Admittedly, great care should be taken in interpreting the observed effects of various detrimental alterations of viral genomes. Such effects may be modified by the presence in the investigated quasispecies populations of minute, hardly detectable (by common sequencing techniques) amounts of different genome variants, which may serve as complementation or recombination partners. For example, the population of *ori*L-truncated CVB3 RNA in human endomyocardial tissue was reported to contain 0.9% molecules with an apparently intact 5′ end (153).

CONSERVATION VERSUS EVOLVABILITY

Evolutionary mechanisms serve two opposite goals: to maintain the stability of viral genomes (to retain their structural and functional identity), on the one hand, and to allow their evolvability (to ensure their capacity to change), on the other. These operate mostly under constant and changed environmental conditions, respectively.

So far, no ancient picornaviral RNA genomes from paleontological or archeological samples are available, and the "ages" of the relevant viral species can be roughly evaluated only on the basis of comparison of nucleotide sequences of the most distant representatives of their genomes. Due to inherent limitations of bioinformatics tools owing to the saturation of the level of nucleotide substitutions and the effects of purifying selection, they may provide more or less reliable estimates only for relatively recent time intervals (up to several hundred or thousand years) (163). Given this limitation, the estimates indicate that picornaviral species may retain their recognizability for at least centuries (164). On the other hand, taking into account the conservation of the key structural features of their virions and some essential nonstructural proteins, there is little doubt that all picornaviruses had a common ancestor in the very far past, i.e., are a monophyletic group whose members diverged through various qualitative jumps (165).

Mechanisms involved in genome conservation and evolvability are considered below.

TYPES AND MECHANISMS OF GENETIC MODIFICATIONS

During their life cycle and evolution, viral RNA genomes should cope with various intrinsic and extrinsic factors potentially capable of disturbing their functions. Adverse phenotypic effects of a plethora of natural or engineered genetic alterations of viral genomes are reported in the literature. Only some illuminating examples are considered here.

Point Mutations

A major source of genetic modifications experienced by the genomes of RNA viruses is the inaccuracy of their replication machinery, caused by the above-mentioned error-prone performance of the viral RdRP and the lack (with the exception of a small set of viruses [see below]) of proofreading capacity. Also, eukaryotic cells possess two families of nucleotide deaminases: double-stranded RNA-specific adenosine deaminases (ADARs), which convert adenosine into inosine (166, 167), and single-stranded RNA-specific cytidine deaminases (APOBECs), which convert cytidine to uridine (168).

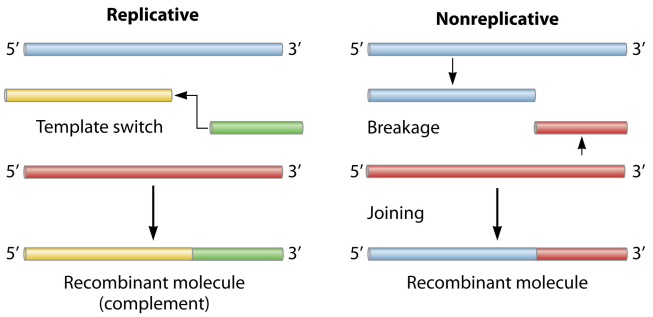

FIG 3 Models of replicative and nonreplicative recombination. See the text for details.

Although the effects of these multifunctional enzymes on RNA viruses are thus far focused mostly on different aspects of virus-host interactions (169, 170), their potential mutagenic effects should not be underappreciated. Such effects of ionizing radiation and other environmental mutagenic factors should not be ignored either.

Insertions, Deletions, and Replacements

An important class of modifications of RNA genomes is represented by their rearrangements, i.e., acquisition of deletions, insertions (including duplications), and replacements of genomic parts by related or unrelated sequences. The rate of generation of insertions/deletions (indels) during a cycle of picornavirus reproduction appears to be comparable to that of the appearance of point mutations, as suggested, for example, by studies on the mechanisms of recovery after adverse alterations of the poliovirus genome (171, 172). Also, ready accumulation of deletion-containing, so-called defective interfering (DI) genomes (see below) under certain conditions demonstrates that such rearrangements are relatively common products of normal reproduction of RNA viruses. Nevertheless, indels are less common in natural viral populations due to their removal by negative selection because of generally strong fitness-impairing effects. These effects may be due to various mechanisms, the most important being interruption (or shifting) of ORFs, destruction of important protein structures and RNA *cis*-acting elements as well as (for long inserts) their A/U content (173), and limitation in the RNA genome length (174–176).

Genomic rearrangements may be caused by intra- or intermolecular recombination, and the latter may involve genomes of different viruses with various levels of relatedness and even cellular RNAs, resulting in such cases in replacements of portions of the viral genomes by foreign sequences. The key role of such replacements in the evolution of RNA viruses is most clearly illustrated by the above-mentioned qualitative differences in the structure of the replicative and translational *cis*-elements (Fig. 2), shared sometimes by viruses of different families (as is the case, for example, with IRES elements of some picorna- and flaviviruses).

There are two fundamentally distinct mechanisms of RNA recombination: replicative and nonreplicative (Fig. 3). The possible existence of these mechanisms was considered long ago, when appropriate tools for their investigation were not yet available (177–179). Further studies demonstrated that RNA viruses exploit both mechanisms (180).

Replicative recombination occurs via template switches, whereby a working molecule of RdRP prematurely terminates its elongation; the newly synthesized, uncompleted chain departs from its template and lands on a new template to serve as the primer for continuation of the synthesis. This is a generally accepted model (54, 181–185), and most recently, it was supported by demonstration of the dependence of recombination frequency on the fidelity of the viral RdRP (12, 186–188).

Nonreplicative RNA recombination implicates joining of fragments originating from distinct viral (or cellular) RNA molecules without involvement of the viral RdRP, though this enzyme is of course required for further copying of chimeric molecules generated thusly. This kind of recombination was first demonstrated to occur in experiments with

pairs of segments originating from poliovirus genomes disrupted in either the 5′ UTR or the RdRP-encoding region (189, 190). Subsequently, it was observed in other picornaviruses (191, 192) as well as in flaviviruses (193–196). As discussed elsewhere (197), this mechanism may also be responsible for certain earlier observed cases of recombination between alphaviruses (198) and rubiviruses (199) (both belonging to the *Togaviridae* family) and hence appears to be a general phenomenon.

It should be admitted that the mechanistic aspects of both types of RNA recombination are as yet purely understood. Both replicative and nonreplicative recombination can generate homologous (precise) and nonhomologous (imprecise) recombinants, but the nonreplicative mechanism is expected to produce predominantly nonhomologous ones. Unfortunately, no tools are currently available to learn which of these two mechanisms is operative during a given case of natural recombination.

Genome Truncation and Disruption

Both termini of viral RNA genomes (and their complementary sequences) are known to be vulnerable targets for the host cell 5′- and 3′-exonucleases (200). Genome truncation is usually accompanied by a more or less marked loss of fitness and is a subject of numerous studies (see below). On the other hand, situations involving disruptions of viral genomes are very rarely investigated directly due to significant experimental difficulties. Yet losses of genome integrity are expected to occur often enough due to several mechanisms, such as activities of defensive antiviral nucleases (e.g., RNase L) and endoribonucleases involved in RNA interference as well as various mechanisms of host mRNA decay, including the nonsense-mediated one (200–202). The involvement of the latter is expected due to the likely presence of stop codons in the viral quasispecies caused by the infidelity of replication. In addition, environmental factors, such as ionizing radiation and alkylating agents, may also lead to RNA genome disruption. UV-induced viral RNA self-cleavage was recently reported (203).

Randomization

Although the randomization of portions of viral RNA genomes is an artificial intervention, the results obtained with the aid of this approach can be quite relevant to the topic of this review. There are different reasons for experiments with selection of viable viruses from pools of viral RNA with randomized segments. If the segments are relatively short, such experiments can reveal structural features required to ensure viability of the virus and affecting its fitness. In other words, such experiments may provide valuable information about the phenotypic effects of a set of point mutations. On the other hand, randomization of larger genome segments can be regarded as just the replacement of a functional part of the genome with irrelevant (but not necessarily inactive [204]) sequences.

(RELATIVE) NEUTRALITY OF VARIOUS MUTATIONS

Although a significant proportion of point mutations in the genomes of picornaviruses (7) as well as various other RNA viruses (205–208) are detrimental, many nucleotide alterations are fitness neutral or exhibit relatively mild fitness defects. This ability to more or less tolerate genetic alterations may stem from different roots. One of the major ones is the degeneracy of the genetic code. Many nucleotide point mutations in the protein-encoding regions are synonymous, and synonymous mutations are often neutral. Remarkably, simultaneous introduction of 1,297 synonymous nucleotide changes into the poliovirus genome did not appreciably change the viral phenotype (53). However, the biological equality of synonymous codons has several limitations.

First, synonymous mutations are not necessarily neutral because of the phenotypic significance of codon (and codon pair) biases, as shown for picornaviruses (50, 51, 53, 209–212), other positive-strand RNA viruses (213–217), and negative-strand RNA viruses (217–223). These biases may be due mainly to the effects on kinetics of translation (e.g., owing to uneven representation of tRNA species corresponding to different synonymous codons and to some features of the ribosome machinery [224]). In particular, the

different rates of reading of synonymous codons affect the dynamics of folding of the generated proteins (225). In addition, synonymous substitutions in the *cis*-acting RNA elements located within the protein-encoding sequences may also exhibit phenotypic effects (86, 138, 147, 157, 226). The possible involvement of some other mechanisms, such as the existence of alternative functional ORFs and the relevant frameshifting signals (78, 227–231) as well as the abundance of CpG and UpA dinucleotides (232–236), should also be taken into account.

Mutational alterations of amino acids (i.e., nonsynonymous nucleotide substitutions) do not necessarily result in changed fitness. Even substantial alterations of the chemical nature of mutated amino acids do not obligatorily cause appreciable phenotypic changes, as exemplified by the outcome for replacements of certain charged amino acids by alanine in the 2C protein of poliovirus (237) or for a Val-to-Arg replacement in the RdRP of mengovirus (238). Similarly, some mutations in the TGK peptide of the *ori*L-interacting motif of $3C^{pro}$ do not exhibit any appreciable phenotypic alterations, at least in *in vitro* experiments (M. A. Prostova, E. I. Smertina, D. V. Bakhmutov, A. A. Gasparyan, E. V. Khitrina, M. S. Kolesnikova, A. P. Gmyl, and V. I. Agol, unpublished data). Even prevention of viral polyprotein cleavage at a canonical site may be relatively well tolerated, as demonstrated with engineered foot-and-mouth disease virus (FMDV) mutants having amino acid substitutions preventing disruption of the bond between the VP1 and 2A moieties normally cleaved by the viral $3C^{pro}$ protein (239, 240). The 2A protein of this virus is composed of merely 18 amino acids, and capsids of the viable progeny of the mutants contained the VP1-2A fusions instead of VP1.

Another important factor contributing to mutational neutrality is the degeneracy of the spatial RNA structure. Regulatory RNA *cis*-elements usually are composed of active parts ensuring specific RNA-RNA or RNA-protein interactions and scaffolding parts involved in retaining these regulatory parts in the appropriate conformation. These scaffolding functions can be fulfilled by oligonucleotide elements with different primary structures. Thus, the secondary structure of the 5′ UTR of the circulating polioviruses may be conserved despite the acquisition of various point mutations. Moreover, even active, ligand-interacting moieties of RNA *cis*-elements can also exhibit some degree of degeneracy, allowing replacement of a nucleotide by another one without an appreciable loss of function. This is true, for example, for the 5′-end-adjacent *cis*-element *ori*L of the poliovirus RNA, which, as mentioned above, interacts with the viral nonstructural protein 3CD. Quite illuminating (and unexpected) results in this respect were obtained upon the randomization of the apical tetraloop and two adjacent base pairs of domain *d* of poliovirus *ori*L (52). These experiments demonstrated that each position in the 39 unique sequences of this octanucleotide in 62 investigated viable plaque-forming viruses could be occupied by any nucleotide (with the exception of one position, which lacked U), though with certain sequence preferences. A closer look indicated that the tetranucleotide corresponding to the loop in nearly half of the isolates fitted the YNMG (Y = U/C, N = any nucleotide, and M = A/C) consensus, and the spatial structures of the relevant tetraloops are known (241). Certain tetranucleotide loops of the genomes of several other isolates had sequences compatible with either a YNUG or GNUA consensus. Some tetraloops with such sequences are known to be able to adopt YNMG-like conformations as well (242, 243). When genomes with various YNMG or YNUG tetraloops were engineered, they (and some genomes with GNUA tetraloops) exhibited wild-type-like phenotypes. Thus, tetraloops able to fold stably into a YNMG-like spatial structure appeared to be well-accepted partners for their protein ligands, clearly illustrating that functional degeneracy of spatial RNA structures may contribute to viral mutational robustness (also see below).

Many experimentally introduced deletions in other parts of the untranslated regions of picornaviral genomes did not result in appreciable functional deficiencies (244, 245). Even the entire deletion of two conserved secondary structure domains of the IRES still did not kill the virus (246).

Various indels in the protein-encoding part of the genome may not be accompanied by marked fitness losses. Thus, engineered insertions of various foreign functional

elements, e.g., antigens (e.g., see references 173, 175, and 247 to 258), tags (e.g., see references 176 and 259 to 264), large structures such as IRES elements creating bicistronic genomes (265–267), or even IRES elements together with sequences encoding additional polypeptides (268–270), may be relatively well tolerated and not infrequently proved to be genetically stable (see below, however). Relatively long deletions in the C-terminal region of the FMDV nonstructural 3A protein were not accompanied by any significant phenotypic alterations, at least *in vitro* (271). Replacements of octapeptides in an antigenically dominant loop of the FMDV VP1 protein by unrelated sequences also produced viable and relatively stable viruses (272, 273).

The occurrence of indels that are not markedly harmful has been documented for noncoding regions of natural picornaviral genomes as well. For example, length differences of up to several dozen nucleotides were registered for the 5′ UTRs (60) and 3′ UTRs (274, 275) of closely related representatives of polioviruses and FMDV, respectively. The indels may not be strictly neutral, but if they are associated with some fitness cost, the detrimental phenotypic changes are likely to be suppressed by some second-site mutations (see below). In some cases, such indels appeared to even be advantageous, judging by their strong conservation in representatives of a given picornavirus species, as is the case, for example, with the triplication of the VPg gene in the FMDV RNA (276, 277) and the duplication of the 5′-terminal *cis*-element of the bovine enterovirus RNA (278, 279). Conserved duplications in untranslated and coding RNA regions of other viruses have been described as well (cf. references 280 to 284).

Notably, replacements of the functional genomic parts by functionally analogous parts of the genomes of not closely related viruses may sometimes also be relatively well tolerated, as exemplified by engineered poliovirus genomes with the IRES of encephalomyocarditis virus (EMCV; a cardiovirus) (117, 268) or human hepatitis C virus (HCV) (285) in place of their own structurally different IRES.

It should again be noted that the attribution of neutrality to specific mutations has to be done cautiously. The true neutrality of a given natural mutation and quantitative estimations of relative fitness are rarely investigated in rigorous experiments. A mutation can be neutral under certain conditions but may be linked to an altered phenotype under others; host-dependent or cell-dependent mutations are good examples of this. Thus, a point mutation ($A_{637}C$) within a double-stranded stem of the type II IRES of Theiler's murine encephalomyelitis virus (TMEV; a cardiovirus), while not markedly affecting the capacity of the virus to grow in nonneural cells, resulted in a several-orders-of-magnitude decrease in its neurovirulence (286). This attenuating effect was due to a decreased affinity of the mutated IRES for the neural form of polypyrimidine tract-binding protein (nPTB) required for efficient initiation of translation of the viral RNA in these cells. FMDV with an extended (>150 nt) deletion in the replication-controlling 5′-terminal hairpin of its RNA did not demonstrate any appreciable phenotypic changes upon infection of cells with deficient innate immunity but proved to be highly attenuated in a mouse model (287). Furthermore, a mutation can be truly neutral within a certain genetic context but may confer an altered fitness within another context owing to epistasis (288). Also, it was demonstrated recently that certain synonymous substitutions, while not immediately changing the viral phenotype *in vitro*, might markedly diminish the mutational tolerance. Thus, the replacement of Ser and Leu codons in some genes of CVB3 and influenza A viruses by synonymous ones more prone to be converted into stop codons by point mutations rendered these viruses more vulnerable to natural and drug-induced mutagenesis (289).

On the other hand, it is not always correct to consider the mutational loss of the capacity to trigger an obvious cytopathic effect (CPE) (e.g., a lack of plaque-forming activity) sufficient evidence for killing of the virus, as usually done. For example, as already mentioned, complete destruction of the CVB3 replicative element *ori*L (99) or *cre* (149), resulting in a loss of cytopathic activity, is nevertheless compatible with viral viability: such viruses are able to grow, though with a greatly diminished efficiency, causing persistent, noncytopathic infections. An FMDV variant that accumulated various debilitating mutations during multiple plaque-to-plaque (bottlenecking) passages

represented another example of conversion of a lytic virus into a noncytocidal one (290).

NATURAL TOOLS FOR CURING DAMAGED RNA GENOMES

Viruses have evolved a number of efficient tools to cope with the potential genetic damages caused by the infidelity of their replicative machinery (as well as by other adverse factors [see references 291 and 292]). One such tool, obviously, is negative selection resulting in the elimination of less-fit variants. However, this mechanism cannot ensure the maintenance of genetic stability upon overcoming various bottlenecks. In these cases, the key roles are played by the rehabilitation tools that are detailed below, but the most general principle is to "fight fire with fire." In other words, the impairments caused by nucleotide substitutions can be counteracted by either reversions or compensatory second-site mutations, owing again to the infidelity of viral RdRPs. More extended genetic alterations, such as indels, can be restored or compensated by intra- or intermolecular rearrangements based on RNA recombination. Of course, recombination may also cure some defects caused by point mutations.

Injured RNA genomes, even if they are dead, as such, may survive for at least some generations due to complementation, i.e., help provided in *trans* by proteins (or RNA *cis*-elements) encoded by their coinfecting viruses. This mechanism of cooperative interaction was described long ago for the case of drug-sensitive and drug-resistant (or -dependent) picornaviruses (293–297), but its wider biological relevance became especially appreciated after the realization that viral populations are represented by quasispecies, i.e., swarms of closely related but distinct individuals (10, 14, 32, 73, 298–301). The prolonged survival of impaired genomes owing to intrapopulation complementation may provide time for their adaptive remodeling, resulting in the restoration of their capacity for independent existence.

REHABILITATION AFTER ADVERSE CHANGES IN THE UNTRANSLATED REGIONS OF THE GENOME

5′ UTR

As mentioned above, the picornaviral 5′ UTRs contain at least two important functional elements, involved in genome replication (*ori*L) and translation (IRES), which exhibit marked structural differences in different representatives of this family. They may be separated from each other as well as from the downstream ORF by spacers with various structures and lengths. Although these spacers usually exhibit a marked level of conservation, their specific functions are so far defined rather poorly. The effects of adverse modifications of distinct 5′ UTR parts are considered separately.

***ori*L.** Generally, enterovirus genomes begin with two unpaired uridines. However, RNAs of CVB3 and poliovirus lacking these 5′-terminal nucleotides were found to be infectious and able to restore their wild-type structure (302, 303). The deleted residues were most likely provided by the VPg-pU-pU primer. However, such a mechanism did not appear to work with similarly deleted RNA of hepatitis A virus (303).

The poliovirus *ori*L can sustain various internal alterations without significant functional impairments. This circumstance endows it with a substantial mutational robustness and creates numerous possibilities for rehabilitation through compensatory second-site mutations should some point mutations inflict an appreciable fitness loss (52). The rehabilitation is, however, not always complete. Although different apical tetraloops of domain *d* potentially able to acquire a YNMG-like conformation are compatible with viability, they endow different levels of fitness. A likely explanation for this phenomenon is the dynamic nature of RNA folding, specifically the instability of a given conformation (caused by thermal motions of constituents) and its existence in equilibrium with others (304, 305). For a given RNA spatial structure, the time of existence in a distinct conformation depends on specific nucleotide sequences. Thus, not all YNMG-like conformations are functionally equal with regard to recognition by their ligands. A factor underlying this inequality is the proportion of time during which the element really adopts the conformation recognizable by its ligand (in this case, the

3CD protein). The fitness of viruses with relatively "poor" YNMG-like folding was enhanced by mutational "improvements" increasing the probability of adopting the necessary conformation (52). Moreover, some tetraloops with known non-YNMG folding (e.g., of the GNRA class) were not lethal but rather quasi-infectious, i.e., no viable viruses with exactly the engineered inappropriate structures could be recovered upon the transfection of susceptible cells, but plaques caused by pseudorevertants did appear upon prolonged incubations. An explanation may consist of the assumption that even these "incorrect" tetraloops (or, rather, their minute proportions) may acquire an acceptable conformation (52, 305, 306). A strong, though not fatal, debilitation of poliovirus caused by inversion of the sequence of stem-loop *b* of *ori*L could be partially ameliorated by spontaneous acquisition of nucleotide substitutions in the loop (117).

Deleterious effects of mutations in the RNA *cis*-elements may also be restored by compensatory mutations in their ligands. As originally demonstrated by Andino et al. (90, 94) and studied in more detail by Prostova et al. (52; unpublished data), harmful alterations of domain *d* of *ori*L may lead to fitness-restoring amino acid replacements in an RNA-binding motif of protein $3C^{pro}$, in particular by changes of the conserved tripeptide TGK into IGK, VGK, and some others.

However, if the original debilitating mutation completely prevents the replicative ability, then there is obviously no possibility for rehabilitation.

IRES. The readiness of reversion of adverse point mutations in the picornavirus IRES was well illustrated by early studies on the instability of the attenuating mutations in this element of the Sabin oral polio vaccine (OPV) strains. In the guts of vaccinees, such reversions occur very often and quite rapidly at positions 480, 481, and 472 in the RNAs of OPV serotypes 1, 2, and 3, respectively (307–311; reviewed in references 312 to 314), leading to restoration of their somewhat impaired secondary (for serotypes 1 and 3) or tertiary (for serotype 2) structures (104, 311, 315) (Fig. 4) and, consequently, translational activity (316–319) and neurovirulence (308, 315, 320, 321).

Similar deattenuating, fitness-increasing effects could be achieved by second-site mutations (pseudoreversions) resulting in the restoration of the impaired secondary or tertiary IRES structure. Wild polioviruses of serotype 1 contain an A_{480}-U_{525} base pair that strengthens a double-stranded element (104) (Fig. 4A). As noted above, the Sabin strain of this serotype has a destabilizing mutation at position 480 (A to G) contributing to its attenuated phenotype, but this destabilization is not usually conserved in vaccinees and their contacts. The strengthening of this base pairing might be accomplished not only by the reversion ($G_{480}A$) but also by a compensating pseudoreversion ($U_{525}C$) (311, 322), which also resulted in a similar increase in virulence (323) and in restoration of the *in vitro* translation efficiency (311). Structural modeling suggested that G_{481} of the predecessor of the Sabin-2 strain, strain P712, could potentially participate in a long-range interaction with C_{398}, supporting the generation of a tertiary structure element (311) (Fig. 4B). On the other hand, the Sabin-2 strain possesses A_{481} and U_{398}, which also can pair with each other, suggesting the functional relevance of this interaction. Nucleotide A_{481} contributes to the attenuation phenotype of this vaccine virus, but it readily reverted to G in organisms of vaccinees, which is the event expected to disrupt the tertiary interaction. However, the potential for formation a stable C_{398}-G_{481} pair was regained in most isolates due to the reversion at position 398 as well (234, 311, 322) (Fig. 4B).

In addition, adverse phenotypic effects (e.g., a temperature-sensitive [*ts*] phenotype) of attenuating mutations in the poliovirus IRES could be partially (and in a cell-specific manner) alleviated by amino acid replacements in the nonstructural 2A protein (324), although the molecular mechanism underlying this improvement is not defined.

Poliovirus IRES (structural type I) (Fig. 2B) also exhibits remarkable plasticity in the response to debilitating indels. Among its several functional elements, there is a tandem of an oligopyrimidine (Y_n) and a cryptic AUG (located at positions 559 to 563 and 586 to 588, respectively, in the Mahoney strain of serotype 1) separated by the 22-nt spacer (171, 325–327). Engineered poliovirus genomes with 23-nt or 39-nt inserts or 8-nt deletions in this spacer proved to be quasi-infectious (171, 172). In the case of

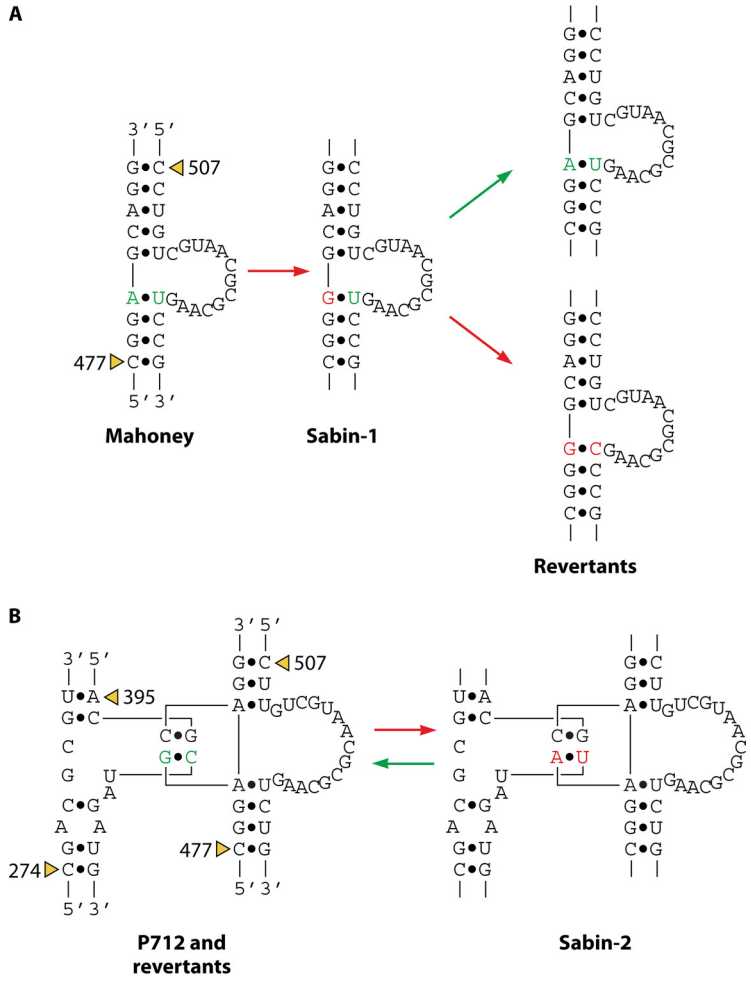

FIG 4 Some attenuating mutations in the IRES elements of Sabin strains and their deattenuation/reversion. (A) Portion of the secondary structure of the IRES of poliovirus type 1. In the parental Mahoney strain, the structure is partly stabilized by pairing between nt A_{480} and U_{525} (marked in green), while it is partially destabilized in Sabin-1 by the $A_{480}G$ substitution (red), contributing to the attenuation of neurovirulence. In the organisms of vaccinees or their contacts, either reversion to A_{480} or the compensatory mutation (pseudoreversion) $G_{525}C$ often takes place, resulting in partial deattenuation. (B) Portion of the proposed tertiary structure of the IRES of poliovirus type 2. The C_{398}-G_{481} interaction (green) in the genome of P712, the wild-type predecessor of Sabin-2, was replaced with U_{398}-A_{481} (red) in the vaccine virus. In the organisms of recipients of the vaccine, the deattenuating replacement $A_{481}G$ usually takes place, accompanied by the reversion at position 398, thereby enhancing the potential for tertiary interaction.

insertions, full or partial fitness restoration was spontaneously achieved in the transfected cells by either deletions resulting in the shortening of the Y_n-AUG spacer to a length close to its natural value or the appearance of a new, noninitiator AUG upstream of the natural and defunctionalized cryptic AUG, again with a more or less "normal" distance from Y_n (Fig. 5). The fitness recovery of genomes with shortened spacers may be accomplished in three different ways: acquisition of an insert (e.g., 9 nt); generation of a new, functional cryptic AUG downstream of the original cryptic AUG and at a comfortable distance from Y_n; or deletion of a large (~150 nt) sequence, resulting in the appearance of a new, comfortably distanced partner for Y_n, the initiator $A_{743}UG$ (Fig. 5). Interestingly, engineered mutant polioviruses with similar extended deletions proved to be markedly attenuated in the monkey neurovirulence assay (328).

One approach to investigating effects of heterologous replacements in the IRES sequence consists of scanning mutagenesis, whereby different adjoining oligonucleotides are replaced by a more or less random oligonucleotide of the same length.

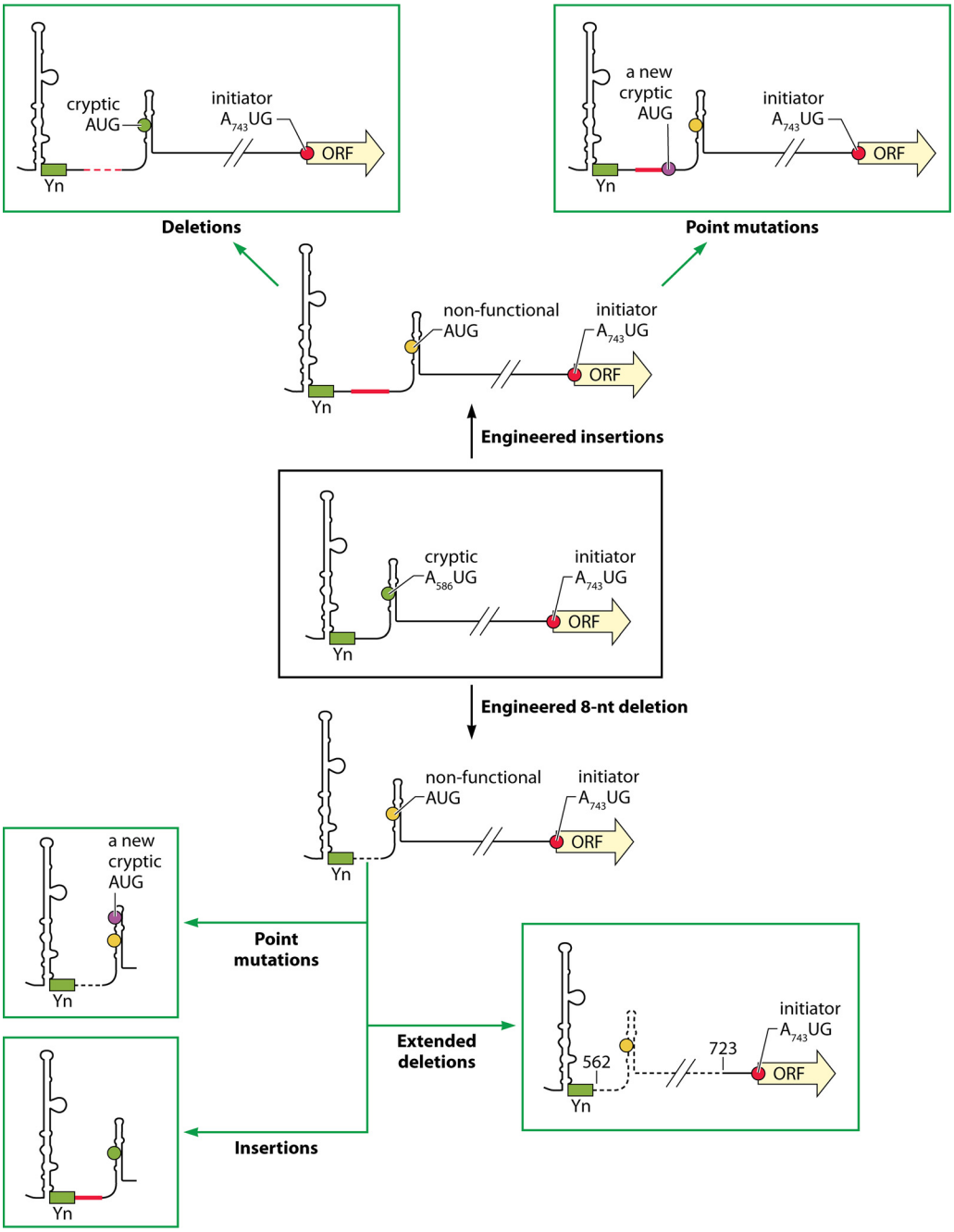

FIG 5 Pathways of rehabilitation after damaging indels in the poliovirus IRES. The wild-type poliovirus IRES (partly presented in the black frame) has a functionally important tandem of an oligopyrimidine (Y_n; green rectangle) and a cryptic (noninitiator) $A_{586}UG$ (green circle) with a spacer of 22 nt. The initiator $A_{743}UG$ is marked as a red circle. Engineering of debilitated constructs and selection of well-fit pseudorevertants are indicated by black and green arrows, respectively. Engineered insertions and deletions are depicted by red and dashed black lines, respectively. Deactivated cryptic AUG and spontaneously acquired functional cryptic AUGs are shown as yellow and purple circles, respectively. See the text for details.

Scanning mutagenesis was used to generate 14 octanucleotide replacements in different loci of the poliovirus IRES (246). Several of them did not kill the virus, though they markedly decreased its fitness. Some mutants exhibited a *ts* phenotype, while some others, though initially considered noninfectious, were in fact quasi-infectious and generated viable pseudorevertants upon further passaging. Their "revival" was due to one or two second-site point mutations in the case of one such mutant and to an

~150-nt large deletion in the downstream region of the 5′ UTR in the case of another (246). The reversions appeared to be host dependent, since the quasi-infectious genomes generated different sets of pseudorevertants in HeLa and neuroblastoma cells (329). The latter observation was likely due to distinctive advantages of certain IRES structures in HeLa and neuroblastoma cells (330–332). Note that the replacement at positions 585 to 592, destroying an RNA helical structure and the cryptic AUG codon, generated a large deletion resulting in creation of a tandem of Y_n and the initiator AUG, similar to that illustrated in Fig. 5.

The rehabilitative capacity of the IRES elements of structural type II was studied less extensively, but the general conclusions were in line with those just described. Destabilization of one of the helices of the IRES of EMCV by a point mutation could be compensated at least partially by acquisition of the true reversion or second-site mutations, particularly (but not only) those that restored the stability of the helix (333, 334). The increased fitness of some pseudorevertants of this mutant could be ascribed to alterations outside the IRES. An apparently compensatory mutation outside the IRES (in the leader protein) was also discovered upon transfection of a genome containing a point mutation in an unpaired segment of the IRES, but explanations for the effects of such second-site mutations are lacking.

Another kind of compensatory mutation concerns the above-mentioned $A_{637}C$ replacement in the TMEV IRES (286). Although this mutation resulted in a strong attenuation of neurovirulence of the virus, intracerebral injections of large doses triggered encephalitis in some mice. The virus isolated from the brains of such animals contained a mutation, $U_{649}C$, in a loop of the same module of the IRES. This mutation increased the IRES-nPTB affinity and restored neurovirulence of the virus (286).

Large insertions between Y_n and the initiator AUG of the TMEV RNA resulted in a significant attenuation of virus neurovirulence for mice (335). The viruses isolated from the brains of animals that received large doses of mutated viruses and did succumb to the disease invariably had acquired either deletions or a new AUG-generating mutation, both adjusting the Y_n-AUG distance to a more comfortable (closer to the wild-type) value, resembling the above-described results with the poliovirus IRES of structural type I.

5′ UTR spacer. The *ori*L and IRES in various picornaviruses may be separated by conserved spacer sequences with poorly defined functions. Their alterations might also lead to adverse fitness effects, which may be ameliorated by second-site mutations. Thus, a 4-nt insert at position 220 of the 5′ UTR of Sabin-1 poliovirus resulted in a small-plaque phenotype, but different large-plaque variants were selected after passages (244). The fitness gain was accompanied in each case by the acquisition of two second-site point mutations in different regions of the 5′ UTR, obligatorily including one of the two above-mentioned deattenuation mutations, at position 480 or 525. A strong *ts* phenotype caused by a 4-nt deletion at the same locus could be ameliorated spontaneously by enlarging this deletion to 41 nt (336).

3′ UTR

***ori*R.** Complete or partial deletions of the picornaviral *ori*R may result in a marked suppression of genome replication at normal or supra-optimal temperatures. These adverse effects may be ameliorated, at least partially, upon passage of the mutants. Thus, an 8-nt insert in the poliovirus 3′ UTR resulted in *ts* viruses, from which ts^+ revertants were selected (337, 338). The phenotypic improvement was accompanied by deletions of 7 or 8 partly original, partly inserted nucleotides (in different isolates, this resulted in four distinct but closely related structures) and by an additional point mutation in some of them. A 14-nt deletion of a stem-loop of the structurally different *ori*R of mengovirus led to a quasi-infectious genome (135). A partial compensatory effect was achieved by an amino acid substitution in the viral RdRP, perhaps through increasing its affinity for *ori*R. If so, this pseudoreversion gives another example of compensation of a mutation in an RNA *cis*-element by alteration of its protein ligand.

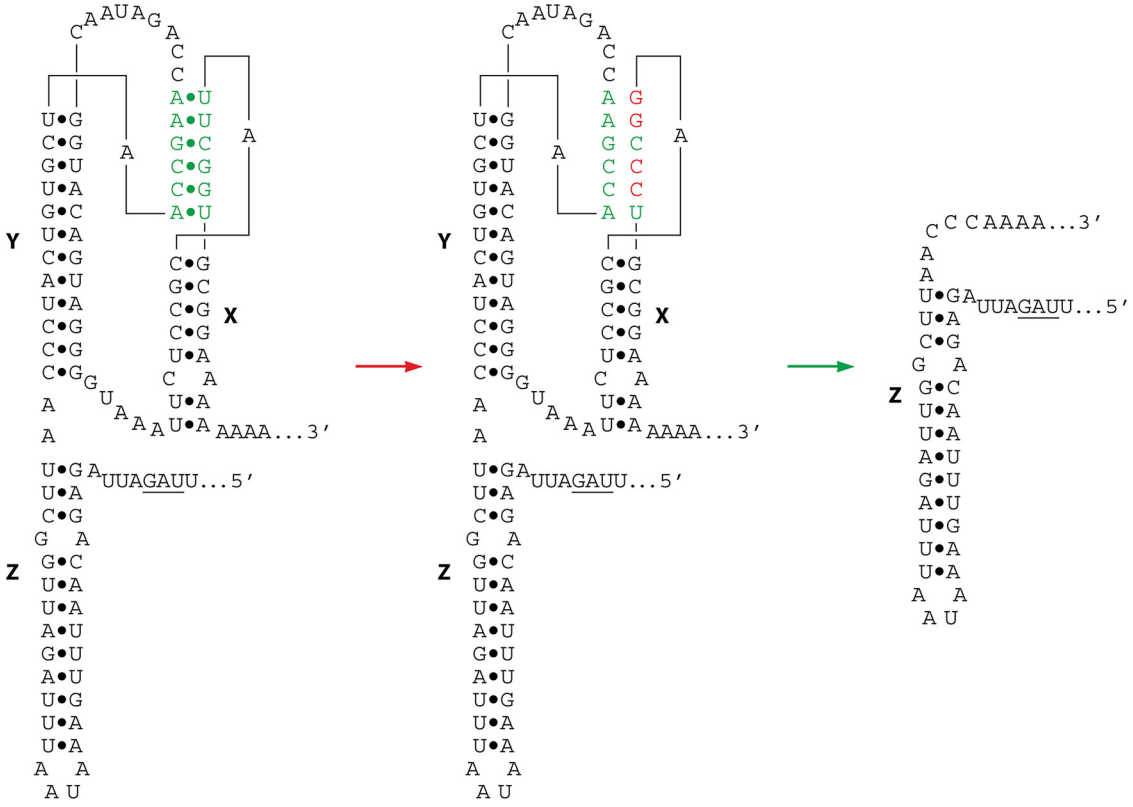

FIG 6 Example of rehabilitation of a picornavirus by the loss of an important RNA *cis*-element. Functional replicative activity of *ori*R of the coxsackievirus B3 genome requires a tertiary (kissing) interaction between its X and Y domains. Mutational alteration of 4 nt in the loop of domain X (shown in red) destroyed this interaction and rendered the viral genome quasi-infectious. A significant fitness gain could be achieved by the spontaneous destruction (rather than repair) of *ori*R through the deletion of domains X and Y. The termination codon of the polyprotein ORF, UAG, is underlined.

An even stronger fitness-increasing effect resulted from natural acquisition of a combination of this RdRP mutation with a mutation at another 3′ UTR position (135).

Debilitating effects of various disruptions of the tertiary kissing interactions between stem-loops X and Y of the coxsackievirus A-9 (CAV9) *ori*R (Fig. 2C) could be compensated, at least partially, by the natural nucleotide substitutions restoring this interaction, including a variant in which the kissing interaction was shifted by 1 nt (120). Interestingly, disruption of certain single base pairs in the kissing interaction of the CVB3 *ori*R may kill the virus (119), whereas more extensive destabilization of this interaction led to quasi-infectious genomes which, after a relatively long period of marginal replication, could increase (though far from completely) their fitness through either a single-nucleotide insertion, allowing an alternative, sufficiently strong kissing, or, unexpectedly, the complete loss of the X and Y domains (124) (Fig. 6).

Poly(A). A set of poly(A)-lacking CVB3 genomes with either plain poly(A) deletion or such a deletion together with removal or randomization of the entire *ori*R, or with the replacement of poly(A) with other homopolymeric sequences, proved to be viable (124). The recovered viruses contained a variety of 3′-terminal sequences, all ending with the regenerated poly(A) sequence. The genome of a virus recovered after transfection with the 3′ UTR-lacking RNA terminated with UAGUCGA$_n$, where the doubly underlined triplet is the translation termination codon of the polyprotein ORF and the singly underlined one is from engineering. Normally, poly(A) is templated by the 5′-terminal poly(U) sequence of the viral negative RNA strand, which in turn is synthesized by copying the poly(A) sequence of the viral genome, but in this case the latter was absent. It is possible that the polyadenylation was accomplished by the terminal adenylyltransferase activity of the viral RdRP, which was demonstrated to exist in the

RdRP of a related poliovirus (339). It also cannot be ruled out that poly(A) was supplied (before the onset of replication?) by a still-undefined cellular mechanism. In any case, the virus was able to survive even such a severe trauma.

An interesting evolutionary trajectory exhibited another engineered genome terminating in the poly(A)-lacking ORF-N_{111}-UCGA sequence (where N_{111} is a randomized RNA segment) (124). The genome of the virus recovered after transfection was terminated with ORF-N_{111}-UCGAG*AAU*$_{13}$*AAUAAA*A_n. Thus, the virus acquired, in addition to poly(A), an AU-rich segment (shown in italics) which contained the AAUAAA cellular polyadenylation signal (underlined) and could be involved in the initial polyadenylation of the engineered genome. The origin of this segment is unknown, but it possibly came from a cellular RNA, e.g., the casein kinase II mRNA, by recombination (replicative or nonreplicative). After further passages, the additional fitness gain was accompanied by further transformation of the genome, which, after 10 passages, acquired a 30-nt segment of the cellular hnRNP U mRNA. Again, recombination was likely responsible for this acquisition. Reappearance of the poly(A) sequence in the engineered tailless genome of hepatitis A virus (having an entirely different *ori*R structure) was also reported (340).

The results described above again illustrate the diverse tools allowing even severely damaged RNA viruses to significantly or completely regain their fitness.

REHABILITATION AFTER ADVERSE CHANGES IN THE INTERNAL REPLICATIVE *CIS*-ELEMENT *cre* (*ori*I)

A marked inhibition of poliovirus and FMDV genome replication caused by disruption of one or two base pairs in the stems of their *cre* elements could be restored by one or two naturally occurring second-site mutations, respectively, resulting in the reestablishment of the helical structure (138, 341, 342).

An exceptional and surprising case of true reversions of a set of 16 point mutations in the *cre* of the engineered RNA of CVB3 was recently published (343). This set was previously reported to be lethal, judging by the inability of the modified genome to induce detectable CPE in transfected cells (147). However, these mutations did not appear to kill the virus, which was still able to replicate in HeLa cells and in some organs of mice, though $\sim 10^5$-fold less efficiently, and was able to trigger persistent (noncytopathic) infection (149). After 8 days of morphologically unapparent reproduction, reversion of all 16 mutations was detected. Strikingly, no intermediates with only some of the reversions could be detected on previous days, implying that only complete reversion endowed the virus with the ability to win the competition with the mutated variants. The genome of the revertant lacked a number of 5′-terminal nucleotides (i.e., part of *ori*L), which were lost before the complete set of reversions had been acquired. The mechanism of the structural recovery in this case is unknown, but the possibility that the reversion occurred through recombination with a cryptic, independently noninfectious but intact-*cre*-element-carrying genome which was present in the quasispecies population should be considered.

REHABILITATION AFTER ADVERSE CHANGES IN VIRAL PROTEINS

The Sabin OPV strains contain attenuation mutations not only in their IRES elements but also in the encoded proteins (321, 344–347). Some of them are known to readily revert in vaccinees or during subsequent transmission, resulting in the restoration of viral fitness (reviewed in references 312 and 313). Since such attenuating mutations are differently located in the genomes of the three OPV serotypes, the possibility exists to replace genomic segments containing such mutations with fitness-enhancing homologous segments from another serotype present in the trivalent OPV, endowing recombinants with selective advantages. Indeed, recombination between OPV serotypes is quite common (310, 348–352). The pattern of distribution of the crossovers in a large set of such intertypic vaccines/vaccine recombinants allowed us to propose the existence of serotype-specific "weak" (fitness-decreasing) regions which are strongly selected against (353). Intriguingly, the locations of these regions may not necessarily

correlate with the locations of the known attenuating mutations, raising questions about the nature of their apparent weakness. It may be added that the Sabin strains can recombine with wild polioviruses and other viruses belonging to the enterovirus C species (354–358), although the resulting fitness alterations are not yet adequately characterized.

An unusual case of reversion of a debilitating mutation in the poliovirus 3AB protein was described by de la Torre et al. (359). The engineered C-to-U transition resulting in the $Thr_{67}Ile$ substitution in this protein was accompanied by acquisition of a strong *ts* phenotype. The reversion of both the nucleotide and the phenotype was accomplished by passaging the mutant at the permissive temperature (33°C). Unexpectedly, three other synonymous mutations (introduced into the investigated genome as markers) also reverted to the wild type in the majority of the revertants. No intermediate genomes possessing only some of these synonymous mutations were detected, to some extent mimicking the above-described collective reversion in the *cre* element. Again, the involvement of recombination with a cryptic wild-type sequence cannot be ruled out.

Structural and functional impairments of a viral protein may be suppressed by second-site mutations. For example, the progeny of a CVB3 genome with an $Asp_{24}Ala$ mutation in the 3A protein acquired either the true reversion or second-site mutations at position 41 of this protein, restoring its dimerization capacity (360). Invalidation of a viral protein can also be compensated by changes in another viral protein involved in the interaction with the affected one. Thus, certain engineered replacements in protein 2C severely suppressed the production of infectious poliovirus due to impairment of the encapsidation mechanism. However, the fitness was spontaneously restored by either second-site mutations in 2C itself or compensatory mutations in its presumptive ligand, the capsid protein VP3 (237, 361, 362). Fittingly, some FMDV mutants with an alteration of the capsid maturation pathway caused by impaired cleavage at the VP1-2A border were reported to acquire mutations in 2C (240).

Still other rehabilitation variants are exemplified by a poliovirus with a 3-nt insert close to the 3′ terminus of the $3C^{pro}$ coding sequence, resulting in apparently complete suppression of viral polyprotein processing at a single cleavage site and rendering the viral genome quasi-infectious (363). Two types of pseudoreversions were detected. The RNA of one recovered virus contained point mutations in both the $3C^{pro}$ and $3D^{pol}$ coding sequences and also lacked the inserted trinucleotide, whereas this insert in the genome of the other revertant was, surprisingly, replaced by a 15-nt fragment of the rRNA.

It was also demonstrated that relatively short fitness-decreasing inserts generated by nonhomologous replicative and nonreplicative recombination could subsequently be removed by homologous recombination (190, 191, 364, 365).

Postdamage rehabilitation may involve one or more intermediates exhibiting different levels of fitness. Thus, a 12-nt insert in the 2C coding region of poliovirus RNA resulted in the acquisition of a *ts* phenotype (366). A pseudorevertant able to grow efficiently at the supra-optimal temperature was shown to have two second-site substitutions in 2C. However, this alteration was accompanied by another phenotypic change: reproduction of the virus became cold sensitive. Remarkably, the harvest of the original *ts* mutant also contained still another pseudorevertant exhibiting temperature dependence similar to that of the wild-type virus. Its 2C coding sequence contained the two mutations present in the cold-sensitive variant and an additional second-site amino acid change, strongly suggesting that it originated from the intermediate cold-sensitive virus (367).

Thus, many adverse amino acid alterations and short indels in poliovirus proteins (and, by implication, those of other picornaviruses) appear to be readily curable by using different rehabilitation mechanisms.

REHABILITATION AFTER LARGE INDELS AND REPLACEMENTS

True spontaneous reversions of large indels may be expected to occur relatively rarely. Nevertheless, an impressive case of very rapid (one cycle of reproduction) and precise deletion of a genomic insertion was observed upon transfection with poliovirus

RNA possessing a tandemly duplicated VPg (3B) gene (368). The specific infectivity of the two-VPg RNA was several orders of magnitude lower than that of its wild-type counterpart, indicating that the insertion was nearly lethal. However, progeny of this debilitated genome exhibited the wild-type (i.e., single-VPg) RNA structure, with the 3′-proximal copy of the VPg gene precisely eliminated, perhaps by homologous intra- or intermolecular recombination. Why just the 3′-proximal copy was deleted is unknown. Interestingly, deletion of "additional" copies of the naturally triplicated FMDV VPg gene resulted, in contrast, in a significant fitness loss or death (369).

Though, as noted above, some engineered insertions encoding various useful experimental tools were reasonably well tolerated and proved to be relatively stable, genetic instability and loss of fitness of viruses with some similar inserts were also demonstrated. For example, insertion of several-hundred-nucleotide sequences encoding green fluorescent protein (GFP) or the Gag protein of human immunodeficiency virus between the 5′ UTR and the polyprotein ORF of poliovirus generated either quasi-infectious or low-fit progeny (174). Similar results were obtained after insertion of the luciferase gene at the same position of the RNA (370). These and some other useful engineered inserts were sometimes lost even upon the first passage. The loss was usually imprecise, resulting in distinct partial deletions suggestive of nonhomologous recombination events, and in certain cases, more than one such event may have been involved. The fitness of the partially repaired populations was markedly increased (though not to the wild-type level) after several passages due to the selection of less-damaged variants. More or less similar results were obtained in other studies as well (257). It should be kept in mind that the expression of a foreign sequence may not necessarily be due to the genetic stability of constructs but may also be due to the intrapopulation complementation of defective genomes (371).

A class of genomes with naturally occurring extended deletions, so-called defective interfering (DI) genomes, has long been known for many RNA viruses (372–374; for some recent references, see references 375 to 385). For poliovirus, DI genomes are present as a minor component in regular laboratory stocks, but their abundance can be increased significantly upon passage at a high multiplicity of infection (MOI). The deletions usually map to the region encoding capsid proteins and may affect a significant portion of this region (386, 387). Although DI genomes are unable to produce viral particles due to a deficit of capsid proteins, they may be endowed with efficient replicative capacity and may not only ensure self-multiplication but also both interfere with viral growth (as their name implies) and provide some replicative proteins in *trans*, thereby assisting reproduction of mutant genomes with impaired replicative functions (371). It is reasonable to assume that similar deletions may spontaneously occur in other parts of the viral genome at comparable rates, but since the resultant viruses are replication deficient, they may escape detection. At least some of them may retain replicative capacity owing to complementation by their coreplicating full-length or defective quasispecies members and thus may participate in genetic exchanges. If so, they may provide an additional source of novel genetic material because they may undergo independent, less-constrained evolution. A remarkable illustration of their evolutionary potential was provided by experimental conversion of FMDV into a virus with a bipartite genome. Multiple ($>$200) tissue culture passages of this virus at a high MOI resulted in the generation of a complex population containing DI genomes with deletions in different genes (388). Due to their mutual complementation, a combination of DI genomes may be propagated at a high MOI in the absence of nondefective helpers, producing lytic infections. Notably, the population with the bipartite FMDV genome demonstrated a higher fitness than that of the parental virus with unsegmented RNA, apparently due to a higher stability of virions encapsidating smaller RNA molecules (389), and certain point mutations accumulated because of the infidelity of RNA replication (390). These observations demonstrated that genome segmentation may represent a mechanism for fitness recovery after genome damages and suggested a model for the origin of picorna-like viruses with a segmented genome (such viruses do indeed exist [391]).

Rehabilitation of fitness impairments caused by deletions in a nonstructural protein may sometimes be accomplished by the acquisition of compensatory mutations in other nonstructural proteins, as exemplified by the restoration of the replicative efficiency of an FMDV mutant lacking a significant part of its 3A protein by a point mutation in 2C (392).

Replacements of genomic regions, which may occur through recombination, are usually regarded as an evolutionary tool for adapting to new or unfavorable conditions or eliminating fitness-decreasing genetic changes (186, 280). However, they may also result in debilitation, and in certain cases such debilitation can be the goal of experimenters. An illuminating example is a poliovirus with the entire IRES exchanged for its counterpart from human rhinovirus type 2 (HRV2) (393). Such viruses, while retaining their poliovirus-like capacity to grow in nonneural human cells, exhibited a strong *ts* phenotype, rendering them highly inefficient at 37°C in neural (and murine) cells (393, 394). As a result, the chimera proved to be highly attenuated with respect to neurovirulence. The marked interest in such viruses is due to their excellent oncolytic properties, making them quite promising tools for treatment of human tumors of glial origin (395, 396). However, passages of these viruses in neural or murine cells under restrictive conditions resulted in partial recovery of their ability to grow at 37°C due to different sets of mutations, typically including 12- or 13-nt deletions just preceding the IRES as well as certain point mutations within the IRES (394). It was suggested that these modifications enhanced the capacity of the IRES to interact with cell-specific ITAFs, thereby optimizing the efficiency of translation.

In contrast to the poliovirus/HRV2 chimera, replacement of the CVB3 IRES with its counterpart from HRV2 did not result in a marked growth deficiency in neural cells (132). This phenotypic difference appeared to depend on the structural dissimilarity of the poliovirus (enterovirus C) and CVB3 (enterovirus B) *ori*R regions (Fig. 2C). When stem-loop Z of the CVB3/HRV2 recombinant was deleted (i.e., when the CVB3 *ori*R was converted into a poliovirus-like one), the capacity of the mutant to grow in neural cells was severely impaired, indicating a functional interdependence of the IRES and *ori*R. However, genetic determination of the phenotypic properties of these chimeric viruses was more complex. When CVB3 lacking stem-loop Z but possessing the HRV2 IRES was passaged in neural cells, its deficiency for growth in these cells was partially ameliorated due to either mutations in the viral nonstructural proteins 3A/3AB or these mutations in combination with mutations in $3C^{pro}$/3CD (397). This observation indicates the existence of a complex network of functional interactions (epistasis) between different parts of the viral genome, i.e., the IRES, *ori*R, and several nonstructural proteins, and the possibility to exploit this network for the rehabilitation of genetic injuries.

A different path of rehabilitation after the damaging effect of IRES exchanges was observed when the CVB3 IRES was replaced by a homologous (and structurally similar) region of echovirus 12. This chimera also exhibited a severe host-specific *ts* phenotype (398). A pseudorevertant of this virus could be selected which regained its fitness through 3 mutations in the IRES, which were suggested to alter its secondary structure.

The above examples illustrate phenotypic improvements of genomes in which exchanges were done between IRES elements of the same structural type (type I). The replacement of the poliovirus IRES by the structurally unrelated IRES of HCV resulted in viable but small-plaque-forming chimeras (285, 399). Passaging one of these viruses resulted in a marked gain of fitness due to selection of mutants with a point substitution or a deletion in the foreign IRES, ensuring its better compatibility with the poliovirus *ori*L (399).

A poliovirus genome in which an extended part of the 5′ UTR (nt 220 to 627) was replaced with the relevant sequence from CVB3 RNA exhibited a *ts* phenotype. However, the wild-type level of fitness was regained through spontaneous deletion of 4 nt (positions 231 to 234, i.e., preceding the IRES) (400). For another chimera, in which a 220-nt 5′-terminal segment of CVB3 RNA was replaced by its poliovirus counterpart, reproduction in HeLa cells was suppressed but was restored after the deletion of a

tetranucleotide in the same locus (positions 232 to 235) (401). This genome also demonstrated a low fitness in simian cells which, however, was improved upon passaging by the acquisition of two additional second-site mutations. The mechanism(s) underlying these impairments/rehabilitations is unknown.

Important results were obtained with engineered mosaic genomes encoding proteins derived from different viruses. A chimera in which the capsid-encoding part (P1) of CAV20 was replaced by its poliovirus analog proved to be quasi-infectious, and its pseudorevertant small-plaque-forming genomes had acquired single point mutations in either the capsid VP3 or nonstructural 2C protein (370). The combination of these two mutations restored the fitness to a nearly wild-type level. The defect in the original chimera was traced to impaired encapsidation of the viral RNA, strongly supporting the role of the VP3-2A interaction in this process (402).

The above examples again demonstrate the multitude of potential trajectories leading to the improvement of the decreased fitness of injured RNA genomes. Although most of these results were obtained by genetic engineering, natural generation of such chimeras is also quite likely.

REHABILITATION AFTER GENOME DISRUPTION

It seems likely enough that fragmentation of viral RNA resulting from either nucleolytic cleavage or incomplete copying is not infrequent during viral reproduction, but there are no reliable tools to detect, let alone investigate, the fate of the relevant fragments. In any case, RNA fragments that are large enough may serve as recombination partners not only to help the recovery of the disrupted viral genomes but also to fuse with sequences coming from different viruses, or even from viral and cellular RNAs (363, 403–405), thereby contributing to viral evolvability.

DEVELOPMENT OF RESISTANCE TO MUTAGENIC AND SOME OTHER INHIBITORS

Although development of viral resistance to inhibitors and rehabilitation after debilitating mutations are formally different topics, the molecular mechanisms underlying these two processes have many common features, since the escape from inhibitory effects of antivirals in some respects mimics restoration of viral functions inflicted by mutations. This is especially true for viral mutagenic inhibitors. Recently, much attention was paid to ribavirin, a purine nucleoside analog which efficiently suppresses a variety of RNA (and DNA) viruses and is widely used in clinical practices (406). A key but not sole mechanism of its activity is its incorporation (after phosphorylation of the respective triphosphate by host enzymes) into viral RNAs by the viral RdRP, mainly in the place of guanosine.

During the synthesis of positive and negative viral RNA strands, ribavirin pairs nearly equally well with cytosine and uracil, resulting in G-to-A and C-to-U mutations, respectively, in viral RNA genomes. Due to accumulation of multiple mutations, incubation of virus-infected cells with this inhibitor may result in complete inactivation of the newly synthesized viral genomes ("error catastrophe") (41, 407–409). However, after multiple passages of poliovirus in the presence of the drug, ribavirin-resistant mutants were isolated, and the mutation responsible for the resistance was traced to $3D^{pol}$ replacements, originally $Gly_{64}Ser$ in the case of poliovirus (8). The resistance was due to a significant increase in the fidelity of the mutated polymerase, which became more reluctant to use the inhibitor as a substrate.

Passaging other picornaviruses, such as CVB3 (410), enterovirus 71 (18), and FMDV (26, 411), in the presence of ribavirin or other mutagenic inhibitors (e.g., 5-fluorouracil [FU] or azacytidine) also resulted in the selection of mutants with altered properties (primarily increased fidelity) of the viral RdRP owing to mutations in different loci of the enzyme. Cross-resistance to these inhibitors was also demonstrated (also see a relevant study with HCV [412]). Thus, the rehabilitation of the functionally inefficient genome in the case of mutagenic inhibitors was facilitated just by their mutagenic effect.

RdRP-dependent resistance to a mutagenic inhibitor can be achieved not only through an increase in the general fidelity of this enzyme but also through more

fine-tuned changes in its properties (413). Viral replication in the presence of the pyrimidine analog FU is usually accompanied by the accumulation of A-to-G and U-to-C transitions. However, a particular FU-resistant (or rather FU-dependent) FMDV mutant encoded an RdRP with a point mutation able to specifically counteract the acquisition of just these transitions without markedly changing the mutant spectrum complexity in the viral progeny.

The acquisition of resistance to a mutagenic inhibitor may also be accompanied by resistance to inhibitors with different modes of action. Thus, a ribavirin-resistant mutant of CVB3 with a point mutation in the RdRP was also resistant to amiloride (410). The latter drug is not mutagenic but rather affects the intracellular ionic environment. It can be concluded that mutations altering RdRP fidelity may also modify some other properties of this enzyme.

Let us briefly consider the acquisition of resistance to some nonmutagenic inhibitors, which is also due to a single or a few point mutations. The above-mentioned guanidine-resistant (g^r) poliovirus mutants are a typical example. The drug targets the viral multifunctional protein 2C (414–418), which possesses an RNA-dependent NTPase activity (419, 420) and, in particular, is hypothesized (though not proved) to function as an RNA helicase (421–423). Both g^r and guanidine-dependent (g^d) classes of mutants display a wide range of phenotypes with different levels of dependence of reproductive efficiency on the guanidine concentration (416, 424). The mutants differ with respect to not only the tolerable concentration of the drug but also whether the virus is truly g^r (i.e., able to grow equally well in the presence and absence of the drug) or g^d (obligatorily requiring the drug for efficient reproduction). As expected, g^r and g^d mutants display some genetic variability within each class. A comparison of our results (418) with those reported by others revealed certain regularities.

The overwhelming majority of mutants with altered guanidine sensitivity possessed one of two amino acid replacements in the 2C protein (never both): either Asn_{179} was changed to Gly or Ala, or Met_{187} was replaced by Leu (for brevity, these mutants belonged to the N or M class, respectively). These mutations are located in the 2C regions thought to be involved in interactions with ATP. Some g^r mutants of the N class and at least one g^d mutant of the M class did not have any other 2C mutations, but the majority of mutants had additional replacements in other regions of this protein, likely associated with phenotype modulations. Some viruses also contained mutations outside 2C. The acquisition by wild genomes of even a single mutation of the N or M class might sometimes require alterations of two nucleotides (due to the properties of the genetic code). Nevertheless, the presence of more than one mutation does not necessarily mean that multiple consecutive steps were required for the acquisition of altered drug sensitivity, because mutants may well originate from representatives of the quasispecies population with sequences different from the master (prevalent) one. In any case, this set of data clearly demonstrates a multiplicity of trajectories which may allow inefficient genomes to reach fitness peaks.

Interestingly, the same point mutation in FMDV 2C ($I_{268}T$) confers resistance not only to guanidine (68) but also to ribavirin, ameliorating the mutagenic effect of the latter (425).

It goes without saying that there are a great variety of viral inhibitors with other mechanisms of action and resistance (for example, see reference 426), but a more detailed consideration of the problem of viral drug resistance is outside the scope of this review.

RECOVERY AFTER DEBILITATING BOTTLENECKING

The quasispecies nature of viral populations predicts that various bottlenecking events, which regularly occur during viral replication within organisms and during interhost transmission (43), may often result in more or less significant fitness losses owing to the probability of the presence of adverse mutations in the transmitted genomes (the Muller ratchet). The rehabilitation of RNA viruses after damaging bottlenecking may exploit all the above-discussed mechanisms of regaining fitness, but

specifically, this phenomenon in picornaviruses was first studied experimentally by multiple consecutive plaque-to-plaque passages of FMDV (427). This procedure resulted in various levels of fitness losses due to different mutations in different lineages. A low fitness of one of the victims of this ratchet was associated with several point mutations scattered over the genome as well as with a significant extension (heterogeneous in length but, on average, 28 nt long) of a penta-adenylate in the N-terminal region of the polyprotein ORF (428). When this invalidated virus and its four plaque-purified subclones were subjected to serial passages in susceptible cells at different MOIs, they regained their fitness to different extents but, remarkably, exploited different pathways to achieve this. The internal A_n stretch of the genome was invariably corrected either by true reversion to the original A_5, by its shortening, or by an extended deletion of 69 nt that included it. In addition, separate lineages exhibited different sets of true reversions and novel point mutations (including single nucleotide deletions). These observations again illustrate how debilitated viruses can reach different fitness peaks by wandering along individual trajectories.

Notably, even more prolonged plaque-to-plaque transmission did not lead to complete extinction of the FMDV populations (290, 429), suggesting that the natural level of replicative infidelity of this virus is finely tuned to ensure prevention of error catastrophe under such conditions.

SOME ADDITIONAL LESSONS FROM OTHER RNA VIRUSES

Positive-Strand RNA Viruses

As we have seen above, a key factor influencing the rehabilitative capacity of damaged picornavirus genomes is the infidelity of the viral replicative machinery. As far as other viruses with positive-strand RNA are concerned, relatively high ($\sim 10^{-4}$) and low ($\sim 10^{-6}$) error rates have been reported for bacteriophage Qβ and coronaviruses, respectively (430–432). These levels may vary even among different representatives of a particular species (433). The variability depends not only on the peculiarities of the RdRPs but also on the properties of other nonstructural proteins. Thus, the fidelity of alphavirus replication is also modulated by mutations in the helicase/protease (nsP2) (434). Remarkably, the *Nidovirales* (with the exception of arteriviruses) exhibit a capacity for correcting replicative nucleotide misincorporation that is apparently unique among RNA viruses and is due to the possession of a 3′-to-5′ exoribonuclease, the nonstructural protein nsp14 (433, 435–438), which functions in cooperation with another nonstructural protein, nsp10 (439, 440). A decrease in severe acute respiratory syndrome (SARS) coronavirus fidelity due to inactivation of this exoribonuclease was accompanied by a significant loss of viral fitness (441).

The recombination frequency varies as well, for example, being relatively high in nidoviruses (442–445) and rather low in flaviviruses (185, 446). Like the situation with picornaviruses, this frequency in other RNA viruses depends on the fidelity of the RdRP (381). On the one hand, low error and recombination rates endow viral genomes with a greater stability, but on the other hand, they diminish their capacity for recovery in the case of damage.

Numerous studies have demonstrated the importance of recombination for the rehabilitation of debilitated positive-strand RNA plant viruses (184), and only some examples are considered here. A segment (RNA3) of the tripartite RNA genome of the cowpea chlorotic mottle virus (a bromovirus) encodes two proteins. Two variants of this segment were constructed, with deletions inactivating one or the other of these proteins. When plants were coinoculated with both deletion variants and intact RNAs 1 and 2, the altered RNA3 was regenerated by recombination (447). The genome of a tobacco etch virus (a potyvirus) with engineered insertions of either its own genes (duplication) or foreign genes (pseudogenization) demonstrated either severe debilitation or even apparent death. However, passages of these low-fit mutants resulted in a more or less rapid enhancement of their reproductive (and competitive) capacity due to the removal of the inserts (448, 449). Inactivating indels in the replicase (i.e., RdRP) gene of phage Qβ could be repaired by homologous recombination, with the intact

gene provided in *trans* by a resident plasmid (450), implying that rehabilitation of even such severe disturbances may occur under natural conditions as well.

In RNA viruses with non-IRES-dependent initiation mechanisms, recovery from significant translational defects may be based on different paths. For example, an engineered 19-nt deletion affecting the hairpins controlling translation of the MS2 phage capsid protein could be ameliorated, upon passages in *Escherichia coli*, by either deletion of 6 more nucleotides, acquisition of an unrelated 18-nt insert, or both modifications, creating novel functional regulatory structures which included the Shine-Dalgarno sequence (451). A special case of mutational robustness was recently discovered in nidoviruses (452). Proteins of these viruses are largely translated from subgenomic mRNAs (sgRNAs), and the synthesis of each sgRNA is controlled by a distinct *cis*-element (TRS). By using deep sequencing, numerous overlapping sgRNAs controlled by different TRS were found to encode a given viral protein. This redundancy ensures continued protein synthesis in the case of mutational inactivation of a TRS.

Various molecular mechanisms are operative in nonpicornaviral RNA viruses to repair or compensate for debilitating alterations of their genomic ends. Different pathways for rehabilitation after adverse modifications of the 5′ UTR were demonstrated for Venezuelan equine encephalitis virus (VEEV) or, more precisely, a chimera of VEEV with Sindbis virus (453). The 3′ end of the viral negative RNA strand (serving as the promoter for the synthesis of the positive strand) has a terminal unpaired dinucleotide and adjoining hairpin with a short C/G-rich stem. Mutations introduced into the similarly folded 5′ end of the positive RNA (into either the unpaired AU end or the hairpin's stem) had significant adverse effects on genome replication, but three classes of pseudorevertants were selected on passaging, containing various single-stranded 5′-terminal extensions rich in AUG or AU repeats, new heterologous stem-loops, or mutations in several nonstructural proteins. Interestingly, compensatory mutations in two nonstructural VEEV proteins were also observed after a detrimental (not fatal) extended deletion of two stem-loops of another *cis*-acting replicative element located not far from the beginning of the ORF (454). Truncation of the 5′-terminal nucleotides of certain plant viruses with positive-strand RNA genomes is also not lethal and can be repaired by various mechanisms, including recombination and nontemplated nucleotide additions by the viral RdRP (455). The RNA of plum pox virus (a potyvirus) is started with A_4. This A_4 sequence is regained after infection with engineered viral RNAs having either one additional A residue or deletion of one or two A residues at this location (456). It was suggested that the correction could occur during the synthesis of the 3′ end of the negative RNA rather than that of the 5′ end of the positive strand, and thus may involve one of the mechanisms of repair of 3′-terminal sequences considered just below.

Various deletions and insertions in the 3′ UTR as well as complete deletion of the poly(A) tail may not kill alphaviruses (457–459), flaviviruses (460–462), coronaviruses (463–465), and various plant viruses (466–469). The repair of the damaged genomes may again involve different mechanisms, including RNA recombination, the use of the viral RdRP- or host-dependent polyadenylation activities, and perhaps some others.

Circularization of flavivirus genomes through interactions between several complementary motifs located at both termini is important for viral reproduction (see reference 470 and references therein). Disruption of some such interactions resulted in a fitness loss, which could be restored at least partially by spontaneous reversions or second-site mutations leading to the restoration/rebuilding of the circularization potential (470, 471).

In positive-strand RNA plant viruses that possess 3′-terminal tRNA-like structures serving as important multifunctional *cis*-elements (472, 473), aminoacylation of this element is involved in translation and/or replication of the genome, and this reaction requires the presence of the 3′-CCA_{OH} end. However, newly synthesized RNA molecules of these viruses often terminate with 3′-CC_{OH}. Repair of the functional structure is likely accomplished by host enzymes, e.g., [CTP, ATP]:tRNA nucleotidyltransferases (474). Various terminal and internal substitutions and deletions in this element may also be

corrected through recombination between distinct components of the multipartite viral genomes, and perhaps by other mechanisms (475–478).

Some plant viruses with positive-strand RNA genomes having non-tRNA-like ends are terminated with the 3′-CCC_{OH} sequence. This trinucleotide and adjacent sequences are important for efficient genome replication, but if modified or even absent, they may be spontaneously restored (479–482). The rehabilitation can be achieved by implementation of different mechanisms (483). In viruses with multipartite RNA genomes (which sometimes have an additional, so-called satellite RNA), the damaged 3′ terminus of a genomic segment may be repaired by making use of another segment either as the recombination partner or as the template for synthesis of a primer for the initiation of the complement of the defective segment. Also, these viruses possess a functionally important stem-loop structure close to the genomic 3′ end. When the sequence of this element was randomized and the resulting RNA was inoculated into susceptible plants, viable viruses with a marked variety of structures of the relevant stem-loop were selected (484). Truncation of 3′-terminal sequences might result in various rearrangements at this end, such as additional deletions or acquisition of some foreign heterogeneous oligonucleotides, apparently arising through nonhomologous recombination with internal parts of the positive or negative viral RNA strand (485, 486).

Negative-Strand and Double-Stranded RNA Viruses

The fidelity of replication of viruses with negative-strand RNA genomes appears to be comparable to that of positive-strand viruses, varying among different representatives of both groups (487), but the former recombine much less often, and for some of them recombinogenic activity was not demonstrated at all (185, 488, 489). This may be explained at least in part by the use of RNP rather than naked RNA as a transcriptional template (490), which is expected to hamper the template switch and some other mechanisms of formation of chimeric genomes. Nevertheless, true intermolecular recombination in these viruses has continuously been demonstrated (491–499). The error rate (487) and frequency of intermolecular recombination of viruses with double-stranded RNA genomes are relatively low due to peculiarities of their replicative machinery, but again, instances of natural recombination between them have been reported (500–503).

An interesting example of regaining fitness after an extended in-frame deletion in the coding region of the genome was documented for a spontaneous mutant of influenza A virus (504). This mutant was missing 36 nt in the RNA segment coding for the NS1 protein and exhibited *ts* and small-plaque phenotypes. Passages of this virus *in vitro* or *in vivo* resulted in the generation of pseudorevertants with wild-type properties, the restoration of which was traced to a single amino acid substitution in the same protein without regaining the lost sequence. It was hypothesized that this substitution permitted generation of an alpha-helix element in the NS1 structure, compensating for the deletion-caused loss of the original alpha-helix.

The deficiency caused by a mutation in one viral protein in some cases can be more or less compensated by alterations in other viral proteins. For example, the matrix (M) protein of vesicular stomatitis virus (VSV) has an anti-interferon activity. Deletion of Met_{51} in this protein rendered the virus interferon sensitive and markedly suppressed its reproduction (505). The fitness was partially restored upon serial passages, and this improvement was attributed to two factors: mutations in another viral protein, phosphoprotein (known to exhibit anti-interferon activity in the rabies virus but not in VSV), and interferon-sensitive members of the quasispecies, which were complemented by their interferon-insensitive counterparts (506). Introduction of 1,378 synonymous mutations in the ORF of the L (RdRP) gene of human respiratory syncytial virus (a pneumovirus) rendered the virus highly temperature sensitive (507). However, passages of its different lineages at stepwise increasing temperatures resulted in the selection of multiple, less-debilitated variants with mutations in various other viral proteins (in 9 of the 11 ORFs) and also in the intergenic regions. The most significant compensatory effects were traced to each of the two alternative amino acid changes in the viral M2-1

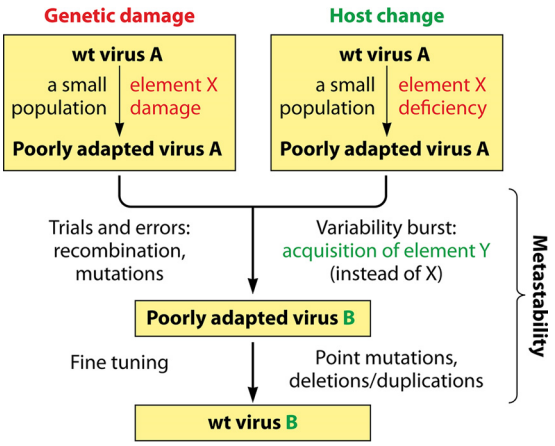

FIG 7 Model of macroevolution of RNA viruses.

antiterminator protein, but the level of rehabilitation could be increased further by combinations of these mutations with alteration in other proteins.

In certain families of viruses with negative-strand and double-stranded RNA (as well as positive-strand RNA), the genomes are composed of several distinct molecules. Such viruses possess an additional rehabilitation tool, reassortment, i.e., exchanges of individual genomic segments between coinfecting viruses (508).

Additional examples of the capacity of RNA viruses to cope with deleterious alterations of their genomes can be found in the review of Barr and Fearns (292).

TO RUN AHEAD, IT IS SOMETIMES USEFUL TO STUMBLE

As we have repeatedly described above, the genomes of RNA viruses are able to maintain their identity under constant conditions despite a significant infidelity of their replicative machinery. In contrast, debilitated genomes are quite unstable. The reason is quite obvious: replication errors are unlikely to increase the fitness of well-adapted viruses but have a much higher chance of being advantageous for weak ones, and the weaker the viruses are, the more different possibilities they have for becoming stronger. To allow them to exploit these possibilities and to fix even slightly advantageous genetic changes, it is necessary not to have more efficient competitors. The invalid viruses very attentively investigate a broad repertoire of options generated by error-prone replication. Point mutations and recombination create a rich swarm of variants, which can follow various evolutionary trajectories. In other words, low-fit viral populations are inherently metastable.

On the one hand, instability opens an array of opportunities for a debilitated virus to regain a wild-type-like genome and thus retain its identity. On the other hand, an important corollary of metastability is a significant potential for the acquisition of qualitatively novel genetic elements, ensuring new phenotypic properties. This constitutes an important basis for viral macroevolution, i.e., generation of novel taxa. Essentially the same events occur if low fitness is caused by changed environmental conditions, e.g., host changes. Indeed, cross-species transmission appears to be a major factor of evolution of RNA viruses (509). A schematic model of these processes is presented in Fig. 7.

Thus, to run ahead, it is sometimes useful to stumble.

ROBUSTNESS, RESILIENCE, AND EVOLVABILITY OF VIRAL RNA GENOMES

The data discussed above illustrate that the maintenance of the genetic and phenotypic identity of RNA viruses, despite the infidelity of their replicative machinery, is principally based on two fundamental properties: robustness and resilience. Robustness can be defined as "the invariance of phenotypes in the face of perturbation" (510). In the coding regions of the genomes, it is based primarily on the codon degeneracy

and neutral character of many amino acid substitutions. The robustness of RNA *cis*-elements is due largely to the degeneracy of RNA spatial structures, i.e., the ability of diverse sequences to maintain, stably or temporarily, similar mutual orientations, as well as the phenotypic neutrality of alterations of certain nucleotides involved in the interactions with specific ligands.

On the other hand, a large proportion of mutations in both coding and noncoding regions of the viral RNA have more or less adverse effects. One may define robustness more broadly and loosely as the capacity to survive in the face of perturbations. As we have seen, certain conserved elements of the IRES and viral proteins can be damaged without a loss of viability. Even all the three key replicative RNA *cis*-elements of picornaviruses (*ori*L, *cre*, and *ori*R) and the conserved poly(A) tail may not be indispensable for viability. However, in apparent contradiction with this fact, various alterations in these elements were reported to be lethal (52, 119, 121, 136, 341, 511, 512). This discrepancy may represent a manifestation of a significant rule formulated by the Russian author Ilya Il'f: "A watchman was known to check passes very carefully, but those who had no passes at all were allowed to go unquestioned and freely." Thus, it may sometimes be better to have no pass (e.g., a *cis*-element) at all than to have a wrong one (513). An explanation of this paradox may consist of the supposition that a complete lack of presumably essential genetic elements may not fully and unconditionally prohibit the relevant wild-type reaction but rather may dramatically decrease its efficiency, directing the process along a different pathway, for example, requiring another protein cofactor or not requiring some normally important protein participants at all. As a result, debilitated viruses can survive. For those with "wrong passes," such pathways may be prohibited.

Numerous tools are at viral disposal for the restoration of diminished fitness, and the relevant capacity may be called resilience (52) or reparability. Rehabilitation can be achieved by either repair of the injured elements or ("plan B") the compensatory modification of another viral element. In both instances, the same mechanisms as those that caused debilitation are implemented, i.e., infidelity of the replicative machinery (mutations and recombination). The fixation of the "cured" genome is achieved by selection.

The resilience mechanisms may serve two opposite evolutionary trends. On the one hand, they may result in the full or nearly full restoration (conservation) of the original genome structure and/or phenotype. On the other hand, they are very powerful factors contributing to viral evolvability. Indeed, stepwise acquisition of small improvements upon wandering along rugged fitness landscapes (514), especially in the absence of stronger competitors, may produce a remarkably broad menu of more-fit mutants able to satisfy various viral "tastes" and hence be a major tool for gaining qualitative novelties. The fixation of the achieved results is done by selection, which in both its forms (negative and positive) is one of the key factors contributing to the conservation and evolvability of RNA genomes.

The relationships between mutation rates, quasispecies, robustness, and evolvability of RNA viruses are discussed in detail in numerous publications (487, 515–526). This problem is closely related to the emergence/reemergence of pathogenic viruses, which has recently become a hot topic (527–532).

Obviously, the regularities discussed in this review are important not only for a deeper understanding of certain fundamental aspects of the lifestyle of RNA viruses but also for numerous applied problems, such as the efficiency of antiviral tools and the development of drug resistance (533–537).

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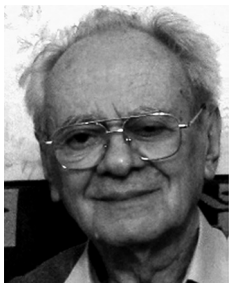

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